

**TITLE OF THE INVENTION**

IMPROVED-BACTERICIDAL AND NON-BACTERICIDAL SOLUTIONS FOR  
REMOVING BIOFILMS

**5 FIELD OF THE INVENTION**

This invention relates to solutions capable of efficiently cleaning surfaces susceptible to biofilm coating thereon. It further relates to a cleaning/disinfecting solution, comprising the cleaning components and a bactericidal effective amount of a disinfectant.

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**BACKGROUND OF THE INVENTION**

Bacteria in natural aquatic environments have the marked tendency to interact with surfaces. The formation of surface biofilms can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients. Bacteria growing in natural environments produce extensive exopolysaccharide (EPS) polymers that mediate both their attachment to surfaces and the formation of microcolonies and, eventually, the generation of biofilms. Biofilms are much more resistant to destruction than planktonic microorganisms. Although the mechanisms of this resistance are poorly understood, EPS are likely to play a role. In addition, biofilm bacteria are substantially resistant to surfactants, biocides and antibiotics. Two problems can arise from the presence of biofilms in a distributing aqueous system. First, the biofilm can clog pipes and tubings or interfere with the proper function of mechanical devices. Second, bacterial populations living in this protected mode of growth produce planktonic cells that contaminate fluids and alter their properties or, in the case of pathogens, can result in food poisoning or infections. It has also been proposed that biofilms could allow the multiplication of microbial pathogens stochastically present in freshwater, as well as providing a mechanism for bioaccumulation of toxic substances. As a result, microbial biofilms constitute major industrial and medical concerns. These concerns are now being realized in the dental profession.

Dentists, dental surgeons and dental hygienists and their patients are well aware of the importance of meticulously sterilizing and disinfecting dental instruments. Indeed, since dental instruments are used directly in a patient's

mouth, sometimes for invasive or surgical procedures, it is of paramount importance to minimize the presence of microorganisms carried by dental instruments. The microorganisms can range from relatively harmless bacteria to dangerous pathogens. Consequently, efforts are deployed to remove 5 microorganisms from dental instruments and from the fresh water lines feeding dental instruments such as air/water seringes, high speed turbines, and ultrasonic scalers, or from saliva evacuation lines. For most hand held dental instruments, thermal sterilization remains one of the best methods for eradicating microorganism. However, thermal sterilization is obviously not 10 practical for the decontaminating of fresh water lines which remain to this date difficult to rid of microorganisms.

It is well known in the dental profession that small diameter pipes carrying fresh water are contaminated by bacteria and other microorganisms contained in the water flowing through them (Barbeau *et al.* 1996). Some of 15 the microorganisms inevitably adhere to the inner walls of the lines and accumulate together with microscopic sediments or other substances into what is commonly known as a biofilm (Barbeau *et al.* 1997). The biofilm quickly and tenaciously coats the inner walls of the lines and becomes a reservoir for the proliferation of microorganisms. Bacterial populations will 20 rapidly reach alarming levels which will also be found in the water discharge from the dental instruments connected to the fresh water line. For example, the average bacteria count in the water discharge of dental instruments is known to be of approximately 200,000 colony forming units per milliliter (cfu/ml) and in some extreme cases can reach 10,000,000 cfu/ml (Barbeau 25 *et al.* 1996).

Jacquelin *et al.* (Path. Biol. 42(5): 425-431 (1994)) disclose compositions comprising a detergent such as sodium dodecyl sulfate (SDS) or sodium deoxycholate (SDC) and a phenolic disinfectant. The solutions are not efficient to remove and/or destroy biofilms as seen from the photographs 30 of Figure 1 and from the concluding remarks of this reference.

Whittaker *et al.* (Appl. and Env Microbiol. 43(3): 395-403 (1984)) disclose a plurality of compositions tested for their cleaning/disinfecting properties against microorganisms. Their best composition was SDS/urea, which was efficient on chlorine-treated osmosis membranes after 11 days of

treatment, which time is far from being a practical cleaning/disinfecting time for dentistry.

European patent publication 109 279 describes a solution comprising a plurality of essential ingredients for sterilizing surgical apparatuses.

5 Although this reference suggests that biofilm decontamination is contemplated, there is no demonstration whatsoever on that specific issue. Moreover, there is no teaching of any subset of combined ingredients which would be capable by itself to remove the biofilm, and optionally, to kill the embedded bacteria.

10 A commercially available mouthwash sold under the trademark PLAX which comprises SDS 0.25%, sodium benzoate 2% and sodium salicylate 0.2%, supposedly helps in removing dental plaque prior to tooth brushing. The efficacy of this solution against biofilms in general is however doubtful given the short time of contact within which dental plaque is to be removed, even when tooth brushing follows.

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20 Patent publication WO 96/20737, assigned to the present proprietor, describes compositions capable of cleaning and disinfecting biofilm-coated surfaces. These compositions comprise SDS 1% - 2%, hydrogen peroxide 5%, EDTA 1%, mandelic and lactic acids in individual 1% concentration or in combined 2% concentration (mandelic acid being a bactericide). They further describe sub-compositions comprising the same concentrations of SDS/hydrogen peroxide/EDTA and SDS/acids. There is no teaching in these publications of compositions which would be different therefrom and still equivalent thereto, and there is no teaching of how specific components 25 attack the integrity of the biofilms e.g. there is no mechanism of action proposed which would lead to establish a generic class of components useful for the purpose of removing biofilms with high efficacy.

30 Another mouthwash has an excellent bactericide activity, that is CEPACOL™, which comprises a low concentration of cetylpyridinium chloride and ethanol. CEPACOL does not remove biofilms but is very efficient against planktonic bacteria. Cetylpyridium chloride (CPC) has been shown as one possible components in the solutions disclosed in WO 96/20737, and it also was used at a low concentration (0.1%). CPC being also a detergent, we hypothesized it could be used at higher concentrations to replace SDS 35 and hydrogen peroxide.

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USP 4,961,923 describes an irrigant to be used in a dental apparatus, which comprises water, ethanol, hydrogenated starch hydrolysate and a surfactant. The surfactant is preferably polysorbate 80. The irrigant may further comprise a disinfectant like cetylpyridinium chloride or other mouth wash-composing ingredients. This patent aims at dislodging bacteria from a dental pocket with mechanical aid and not at removing well-established biofilms and this, without necessitating any mechanical aid. A composition comprising organic salts or salt forming acids in combination with detergents is not taught in this patent. Further, this document teaches the use of ethanol and surfactants solely for the purpose of dissolving the components, and not for their biofilm dislodging or bactericidal properties.

USP 3,941,696 describes the use of cetylpyridinium chloride as a disinfectant. There is no teaching of a composition which would be capable of dislodging established biofilms.

USP 5,948,390 describes a mouthwash comprising about 0.01%-1% by weight of hydrated uncomplexed zinc cations, about 0.01%-4% by weight of fully or partially protonated citrate moieties; about 0.01%-2% by weight of cetylpyridinium moieties, and an orally acceptable vehicle. This mouthwash composition is formulated at pHs of 3.0 to 4.5. This patent focuses on the addition of a zinc component in a CPC-comprising mouthwash. This patent aims at adding zinc for its anti-microbial and deodorizing properties, while solving the problem of zinc unpleasant and astringent taste. The addition of protonated citrates appears to prevent zinc complex formation, at pH of 3.0 to 4.5.

There still remains a need for compositions for cleaning biofilm-coated surfaces which will effectively dislodge a biofilm and optionally kill the microorganism flora in the dislodged biofilm, these compositions being adapted upon a variety of industrial uses and needs.

### **30 SUMMARY OF THE INVENTION**

Against all expectations and documented evidence, the present inventors found that effective removal of biofilm may be achieved, using a solution minimally comprising a detergent and acids which, at the working pH, form salts in a substantial proportion. These two components by themselves are sufficient to remove well-established biofilms in a period of

time varying from within 1 hour to an indefinite time, more preferably between about 1 hour and 18 hours.

When destruction of microorganisms is a concern, particularly in the medical or dental professions, a bactericide must be added to the solution.

5 The bactericide contacts the surface rid of biofilm and wherein residual microorganisms retained on the surface will be killed. Preferably, the disinfecting and cleaning actions are allowed to occur concurrently.

In accordance with the present invention is provided a solution for dislodging a biofilm from a surface, which comprises an effective dislodging 10 amount of a detergent and an effective dislodging amount of a salt or of an acid which forms a salt at a working pH value, or both, said salt being capable of displacing divalent cations present in the structure of the biofilm with the proviso that the composition is neither a mixture of SDS 1% - 2% and EDTA 1%, a mixture of SDS 1% - 2% and mandelic and lactic acids, 15 each at an individual concentration of 1% or in a combined concentration of 2%, a mixture of SDS 0.25%, sodium salicylate 0.2% and sodium benzoate 2% (PLAX), nor a mixture of 0.1 - 0.3% SDS or SDDD, 0.1 - 0.3% SCS or SLS, 0.1% zinc sulfate, acetate, nitrate or gluconate salts and 0.1 - 0.3% HEEDTA, EDTA or DTPA (EP 109 279), all percentages representing final 20 weight per volume concentrations.

It is another object of this invention to provide a composition for dislodging and destroying a biofilm, which further comprises a bactericidal amount of a bactericide.

In preferred embodiments, the detergent is SDS in a concentration of 25 at least about 0.1 % or any detergent having a biofilm dislodging potency substantially equivalent thereto. The acid is mandelic acid in a concentration of at least about 0.1 % at a working pH value (pH 5 is one example), or a mandelate salt, or any acid or salt having a biofilm dislodging potency substantially equivalent thereto at a suitable working pH. For example, the 30 salt or acid may interestingly be an EDTA salt or acid in a concentration of at least about 0.25 % at a working pH value. At pH 5, EDTA acid forms EDTA salt and is performing when combined to SDS, with or without any other acid, although better results were obtained with another acid.

In more preferred embodiments, the acid is selected from the group 35 consisting of mandelic, 2-ketoglutamic, acetic, iminodiacetic, mucic, glycolic,

fumaric, lactic, aspartic, phosphoric, pyruvic, chloroacetic, oxalic, citric, oxamic, malic, dichloroacetic, phenylacetic, benzylic, maleic, succinic, chloromandelic, glutamic, nitrilotriacetic, boric, adipic, formic, glucuronic, salicylic, benzoic, benzoyl formic, phthalic, ketopimelic acids, alanine, serine, 5 tryptophan, tyrosine, bicine, tricine and glycine. When a bactericidal activity is needed, a bactericide such as hydrogen peroxide or any bactericide having a bactericidal potency substantially equivalent thereto may be added. Other bactericides like phenol derivatives or sodium hypochlorite are examples of good bactericides. They have been used in concentrations of at least 0.1% 10 and 0.5 %, respectively. In even more preferred embodiments, the composition further comprises biofilm dislodging enhancer agents such as chaotropic agents or calcium chelators.

A calcium chelator such as EDTA, preferably in a salt form, in a concentration of at least about 0.25 % or any calcium chelator having a chelating potency substantially equivalent thereto may be added. 15

A chaotropic agent such as SDS in a concentration of at least about 0.1 % or any chaotropic agent having a chaotropic potency substantially equivalent thereto may also be added.

In more preferred embodiments, the compositions comprise at least 20 about 0.1% SDS, at least about 0.1% acid, at least about 0.25% EDTA, the acid being selected from the group consisting of 2-ketoglutaric, acetic, iminodiacetic, mucic, glycolic, fumaric, aspartic, phosphoric, pyruvic, chloroacetic acids and alanine.

In a mostly preferred embodiment, the compositions comprise at least 25 about 0.1% but less than 1% SDS, about 0.1% - 2% acid, and at least about 0.25% but less than 1% EDTA, the acid being mandelic acid or any other of 2-ketoglutaric, acetic, iminodiacetic, mucic, glycolic, fumaric, aspartic, phosphoric, pyruvic, chloroacetic acids and alanine.

The highest concentrations confer a strength to the composition such 30 as it is effective within one hour. The lowest concentrations confer a good performance within 18 hours.

Good bactericides comprise mandelic acid 1%, hydrogen peroxide about 5%, or phenol derivatives at least about 0.1%, or sodium hypochlorite at least about 0.5% These bactericides are tuberculocides e.g. they are

active against *Mycobacterium* spp. which are resistant to a large panel of bactericides.

We have further found that cetylpyrinidium moieties such as chloride or bromide (CPC or CPB) were very good biofilm dislodgers and bactericides  
5 at a concentration higher than 0.5%, when combined to EDTA 1% and a salt or a salt-forming acid 1%, at pH of 7.5. The tuberculocide activity was very good with mandelic acid 1%.

10 **DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION**

Each component tested in this application will be given an abbreviated name, which complete definition is as follows:

SDS	Sodium dodecyl sulfate
EDTA	Ethylenediamine tetraacetic acid
15 H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
CPC	Cetylpyridinium chloride
CPB	Cetylpyridinium bromide
Tween 20	Polyoxyethylene sorbitan monolaurate
SCS	Sodium cocoyl sarcosinate
20 SLS	Sodium lauryl sarcosinate
SDDD	Sodium n-decyl diphenylether disulfonate
HEEDTA	N-(hydroxyethyl)ethylenediamine triacetic acid
DTPA	Diethylenetriamine pentaacetic acid

Starting from the solutions already described in the patent publication  
25 WO 96/20737 assigned to the same proprietor, comprising SDS 1% - 2%/mixture of mandelic and lactic acids 2%/EDTA 1%/hydrogen peroxide 5%, we first replaced mandelic and lactic acids with a plurality of acids used individually in 1% concentration (w/v), the pH of the working solution being brought to 5.0. We further tried different components with hope to find  
30 equivalents for each other essential and non-essential ingredients of the composition.

The compositions were allowed to contact biofilms for 1 and 18 hours to evaluate their cleaning and disinfecting efficacy.

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**Testing the disinfectant.**

**Optical and scanning electron microscopy.**

Two-cm long pieces of dental unit waterlines tubings were used. These tubings were taken from functional dental units installed at the faculty of dentistry of the University of Montreal. Our previous studies (1992-1996) have shown that the lumen of these tubings is covered with mature biofilms. The pieces were sectioned longitudinally with a sterile scalpel blade to expose the biofilm. Another series of tubings was left untouched. Sections of tubings were placed in sterile 5ml disposable test tubes containing solutions to be tested.

After 1 or 18 hours without agitation at room temperature, tubings were rinsed three times with sterile water. Examination was done first with a binocular microscope at a magnification of 40X. Data were recorded on a arbitrary scale by two different examiners and noted as from 4+(same as control) to 0 (no biofilm).

Selected pieces of tubings were processed for electron microscopy.  
*Scanning electron microscopy (SEM)*

For SEM observations, following fixation, post-fixation and dehydration, samples were critical point dried with carbon dioxide in a Balzers CPD 030 Critical Point Dryer (Balzers, Furstentum, Liechtenstein), then mounted with a conductive carbon paint on aluminum stubs and sputtered with gold, or carbon-evaporated in a Bal-Tec MED 020 High Vacuum Coating System (Bal-Tec Products Inc., Middlebury, CT, USA). The interior of tubing segments was examined with a field emission JEOL JSM 6300F SEM operated at an accelerating voltage of 15 kV. The gold-coated specimens were used for SEI while the carbon-coated ones were visualized by BEI.

**Antibacterial activity.**

To test the antibacterial activity, we used the same setup as above. After the 1 or 18 hour incubation, tubings were rinsed with sterile water. Pieces of tubings were dropped in sterile test tubes containing 4 ml of R2Am medium. Test tubes were capped and incubated for 7 days at room temperature. These conditions proved to be best for the growth of the majority of dental unit waterlines bacteria. Data were recorded as presence or absence of growth with a spectrophotometer at  $\lambda=590$  nm.

We have isolated 30 strains of DUWL bacteria among which, *P. aeruginosa*, *P. putida*, *M. mesophilicum*, *A. calcoaceticus*, *P. fluorescens* were the most frequent species. We tested these strains individually in the disinfectant. Pure culture of bacteria in liquid R2Am broth were used.

5 **In situ testing.**

The disinfectant was tested in the ACCM® prototype in a closed room at the Faculty. The ACCM was installed on a A-dec dental unit by one of our technicians. Before the study, water samples were taken for bacterial counts, and a two-cm piece of the air/water syringe hose was taken for SEM. The 10 lines were filled with disinfectant containing alizarin green as an indicator and the setup was left unused overnight. The next day, the disinfectant was drained until no coloration was seen. Draining was done for an extra 2-min and a 4-ml water sample was taken for bacterial counts. Another 2-cm piece of tubing was sectioned for SEM. A second sample was taken at the end of 15 the day and the lines were filled with disinfectant for 18 hours. This routine was repeated over a period of one month. In some experiments, the disinfectant was left to react for 1 hour in lieu of 18 hours.

**Collection and plating of water samples.**

20 All the water samples were vigorously agitated with a vortex for 15 seconds. The plating was done by inoculating Petri dishes with 100 µl of a 1:10, 1:100 and 1:1000 dilution in duplicate, or by using an automatic spiral plating system (Meyer Service & Supply, Ontario, Canada) after a tenfold dilution of the sample. The enumeration was done using a magnifying glass and a counting grid.

25 Control samples (20 ml) were obtained from nearest taps in each clinic and at the source upstream to the connection to the dental unit in selected units. These samples were filtered through a 25 mm polycarbonate filter (0.22 µm) (Millipore, Montréal, Canada) using a sterile syringe and a filter holder (Millipore). The filters were then placed on the surface of the 30 culture medium in a Petri dish and incubated.

Newly installed dental units (Kavo, Germany) at the dental school were also sampled just before their first clinical use with the same sampling technique.

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**Culture conditions.**

A modification of the medium of Reasoner (termed R2Am) was used. The composition is as follows: starch 0.5g, yeast extract 0.5g, trypticase peptone 0.5g, dextrose 0.5g,  $K_2HPO_4$  0.3g,  $MgSO_4$  0.05g, succinate 0.25g, 5 casamino acids 0.5g, agar 7.5g, and distilled water to 1L. Tryptone soy agar and Sheep blood agar (Difco, Montréal, Canada) were also used. Bacteria were cultivated in aerobiosis and anaerobiosis (10%  $CO_2$ , 10%  $H_2$  and 80%  $N_2$ , anaerobic cabinet: Forma Scientific, Montréal, Canada) for the determination of their dependency on oxygen and at 25°C and 37°C over 10 time between 24 and 480 hours.

**Towards the establishment of a quantitative assay**

A more quantitative assay has been built as follows: water samples were collected from dental units and were two-fold diluted with a R2Am medium (which composition per liter is:)

15 1 g soluble starch (Sigma # S-9765)  
1 g yeast extract (BBL 11929)  
1 g trypticase-peptone (BBL 11921)  
1 g dextrose (Difco Laboratories # 0155-17-4)  
0.1 g magnesium sulphate anhydride (Sigma # M-7506)  
20 0.5 g succinic acid (Sigma # S-9637)  
1 g casamino acid (Difco Laboratories (0230-01)  
0.6 g potassium phosphate diabasic (Sigma # P-3786).

The medium was autoclaved for 20 minutes at 121 °C.

25 250  $\mu$ l of the diluted mixture were distributed in each well of a 96-well plate. The plate was incubated at ambient temperature for 2 days. 100  $\mu$ l were withdrawn from each well, and daily replaced with 100  $\mu$ l fresh medium, during four days. Biofilms representative of those growing and coating the dental water line thus formed in the wells. The solutions to be tested were left in contact with the biofilms for 1 to 18 hours. The solutions were withdrawn 30 and the wells were rinsed. The presence of bacteria remaining in the wells, if any, was revealed with violet crystal dye coloration for one hour. The dye was withdrawn and the wells were rinsed. The dye extracted in 300  $\mu$ l of a mixture of 40% methanol (v/v) and 10% acetic acid (v/v). The color intensity was read by spectrophotometry at 570 nm on 100  $\mu$ L aliquots. The 35 absorbance correlates with the residual quantity of biofilm.

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**Acid Substitutions:**

We first substituted a plurality of acids for mandelic and lactic acids. All these acids were used in 1% (w/v) final concentration. Results that have been obtained with the semi-quantitative assay are shown in Table 1.

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Scale evaluation :

- : no biofilm and ++++: no significant removal of film.

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TABLE 1: ACIDS SUBSTITUTING FOR MANDELIC OR LACTIC ACIDS

		DESCRIPTION				RESULTS AFTER DISINFECTION		
		H <sub>2</sub> O <sub>2</sub>	EDTA	SDS	pH	1 hour	18 hours	Growth
5	Selected Acid (1%)							
	Mandelic Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	Lactic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	D-Tartaric acid	5%	1%	1%	5,0 (NaOH 10%)	++	++	-
	Citric Acid	5%	1%	1%	5,0 (NaOH 10%)	+++	+	-
	Oxalic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	+	-
10	Oxamic Acid	5%	1%	1%	5,0 (NaOH 10%)	+++	+	-
	Sulfamic Acid	5%	1%	1%	5,0 (NaOH 10%)	+++	+	-
	Malonic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	++	-
	Malic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	Dichloroacetic Acid	5%	1%	1%	4,99 (AcOH)	+	+	-
15	2-Ketoglutaric Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	Maleic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	Succinic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	Acetic Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	Phenylacetic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	+	-
20	R-Chloromandelic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-

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DESCRIPTION						RESULTS AFTER DISINFECTION		
	Selected Acid (1%)	H <sub>2</sub> O <sub>2</sub>	EDTA	SDS	pH	1 hour	18 hours	Growth
5	L-Serine	5%	1%	1%	5,0 (AcOH)	+	+	-
	D-Phenylalanine	5%	1%	1%	5,0 (HCl 10%)	++	++	-
	Glutamic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	R-2-Phenylglycine	5%	1%	1%	5,0 (AcOH)	++	++	-
	Glycine	5%	1%	1%	5,0 (AcOH)	++	++	-
	Benzilic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	+	-
	Iminodiacetic Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	Nitrilotriacetic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-
	Tricine	5%	1%	1%	5,0 (AcOH)	+++	+	-
	Bicine	5%	1%	1%	5,0 (AcOH)	++	+	-
	Mucic Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	L-Aspartic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	-	-
	L-Ascorbic Acid	5%	1%	1%	5,0 (NaOH 10%)	++++	+++	-
	Phosphoric Acid (H <sub>3</sub> PO <sub>4</sub> )	5%	1%	1%	5,0 (NaOH 10%)	++	-	-
	Boric Acid (B(OH) <sub>3</sub> )	5%	1%	1%	4,99 (AcOH)	+	+	-
10	Pyruvic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	-	-
	Glycolic Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-
	Adipic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-

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	DESCRIPTION					RESULTS AFTER DISINFECTION		
	Selected Acid (1%)	H <sub>2</sub> O <sub>2</sub>	EDTA	SDS	pH	1 hour	18 hours	Growth
Formic Acid	5%	1%	1%	5,0 (NaOH 10%)	+++	+	-	-
Glucuronic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-	-
Salicylic Acid	5%	1%	1%	5,0 (NaOH 10%)	++	+	-	-
Benzoic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-	-
Benzoylformic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-	-
Chloroacetic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	-	-	-
Phthalic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-	-
Fumaric Acid	5%	1%	1%	5,0 (NaOH 10%)	-	-	-	-
Ketopimelic Acid	5%	1%	1%	5,0 (NaOH 10%)	+	+	-	-
L-Tryptophan	5%	1%	1%	5,0 (AcOH)	+	+	-	-
DL-Alanine	5%	1%	1%	5,0 (AcOH)	+	-	-	-
DL-Tyrosine	5%	1%	1%	5,0 (AcOH)	+	+	-	-

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To our surprise, all the tested acids manifested a capacity to dislodge biofilms, leaving the biofilm constituents free for the bactericidal activity of hydrogen peroxide. Some acids conferred a slow but nevertheless significant tendency of the solution to dislodge biofilms with time, while the others were 5 more rapid in this respect. We have selected cleaning solutions capable of performing at practical times: 1 hour or less, and overnight (about 12 - 18 hours). From the above list, seven acids were performing within less than 1 hour: mandelic, 2-ketoglutaric, acetic, iminodiacetic, mucic, glycolic and fumaric acids. To this restricted list of very performing acids can be added 10 those acids performing between 1 and 18 hours: lactic, aspartic, phosphoric, pyruvic, chloroacetic acids and alanine. Since the above results are of semi-quantitative nature only, we add to the list of acceptably performing acids: oxalic, citric, oxamic, malic, dichloroacetic, phenylacetic, benzylic, maleic, succinic, chloromandelic, glutamic, nitrilotriacetic, boric, adipic, formic, 15 glucuronic, salicylic, benzoic, benzoylformic, phthalic, ketopimilic acids as well as serine, tryptophan, tyrosine, bicine, tricine and glycine, because they all led to sufficient decontamination within the specified times. All the other acids can be used when time is not a major concern or when mechanical brushing is used to accelerate removal of biofilm. It is worthwhile noting the 20 isomeric form of an acid does not appear to influence its dislodging capabilities.

Taking the results of acid substitution altogether, it appeared clear that the common point between the acids was that they may form salts at the working pH of 5.0.

25 These observations led us to appreciate why the sub-compositions already published by the present assignee minimally comprising SDS/ acids were performing cleaning and disinfecting solutions. The presence of the detergent and salt forming acids is most probably responsible for breaking the integrity and dislodging the biofilm. Since mandelic acid is also a 30 bactericide, the combination SDS/ acids was also an efficient detergent/ disinfectant solution.

Further, the other sub-compositions disclosed in the same patent publications minimally comprising SDS/EDTA/hydrogen peroxide, which were also performing sub-combinations revealed that EDTA used as a salt was

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capable of complementing SDS in the cleaning component of the solution. Hydrogen peroxide had the role of the bactericide.

It is therefore an object of this invention to provide a minimal cleaning solution comprising a detergent and a salt forming acid (direct addition of salts in the solution is also an option). The bactericides are added in the solutions, when a bactericidal complement is desired. Any bactericide may be added to the above cleaning solution, which would have for effect to confer an additional disinfecting action thereto, which action is greatly facilitated by the dislodging action of the cleaning ingredients: detergent and salts. Amongst the bactericides tested, sodium hypochlorite, phenol derivatives and hydrogen peroxide showed a broad host killing activity, even against *Mycobacterium* sp. known for their high level of resistance towards bactericides. Mandelic acid has a dual role as a salt forming acid and as a bactericide. Povidone-iodine was also tested and had a significant efficiency when combined to the detergent Tween 20<sup>TM</sup>. Cetylpyridinium chloride and bromide have a dual role as a detergent and a bactericide. All the above bactericides are non limitative examples of bactericides.

Equivalents:

The above Table I shows that many acids are equivalent to mandelic and lactic acids.

Different types of detergents have been tried, a cationic one (cetylpyridinium chloride (CPC) and bromide (CPB), also bactericides), as well as hexadecyltrimethylammonium bromide (CTAB) and benzalkonium chloride, non-ionic ones (Tween 20<sup>TM</sup>, MEGA-8 (octanoyl-methyl-glucamide) (O-3129), MYRJ-45 (polyoxyethylene ester-8-stearate) (P-3315), Polidocanol (polyoxyethylene-9-lauryl ether) (P-9641), Tergitol NP-40 (polyglycol ether) (NP-40), Triton X-100 (octylphenoxy-polyethoxyethanol), Tween-85 (polyoxyethylene sorbitan trioleate) (P-4634), Tyloxapol (polyoxyethylene ether) (T-0307) and povidone-iodine, also a bactericide), anionic ones (SDS, SCS and SDDD, alginic acid high and low viscosity (A-7128), Cholic acid (C-1254) and Lithium docecy1 sulfate) and zwitterionic ones (CHAPSO (C-3649)(3-((3-cholamidopropyl)dimethyl-ammonio)-2hydroxy-1-propanesulfonate) and SB3-10 (alkyl-dimethylammonio-propane sulfonate)). These detergents were capable of dislodging biofilms to various extents. SDS, CPC and CPB were the preferred ones. SDS achieved very good

activity even at a concentration as low as 0.15% after one hour, and a perfect efficiency at the same concentration after 18 hours. It is worthwhile noting that the solution tested with 0.15% SDS also contained 6% hydrogen peroxide, and low amounts of HEEDTA (acid; 0.3%), acetic acid (0.1%) and 5 zinc sulfate heptahydrate (0.1%). This solution corresponds to the best one described in EP 109 279. The pH of the solution was adjusted from 2.42 to pH 5, which entails of salt formation. Our minimal solutions would comprise a detergent and salts. As a first optional goal, they would also comprise a bactericide. CPC and CPB fulfilling both detergent and bactericide sought 10 activities, they became our best candidates as detergents/bactericides.

Further, the commercial mouthwash PLAX™, comprising 0.25% SDS, 2% sodium benzoate and 2% sodium salicylate (pH 7.35) showed good biofilm removal, although not perfect, after 18 hours of contact. The acceptable performance of that solution confirms that salts only may be used 15 in lieu of acids.

The above results show that the concentration of detergent and salts may be quite low if the time of contacting is longer (for example overnight), while higher concentrations confer more strength and decrease the time necessary for dislodging biofilms (within 1 hour for example). 20

**Enhancers:**

As mentioned above, we already described sub-combinations which were as efficient as the complete combination SDS 1% - 2 %/EDTA 1%/hydrogen peroxide 5%/mandelic and lactic acids 2%. These sub-combinations comprise the ingredients SDS/EDTA/hydrogen peroxide and 25 SDS/acids. In both cases, sub-compositions comprise the ingredients detergent and bactericide. What was not explained at the time was why EDTA was essential to the first sub-composition to perform well. The above results provide such an explanation: EDTA salts greatly improve the dislodging or cleaning capacity of the solutions. The presence of EDTA is no longer deemed necessary to present cleaning solutions, since EDTA may be 30 replaced by other salts or salt forming acids. EDTA is rather considered as an activity enhancer, because this compound is also a good divalent ion chelator, and as such, it may help in withdrawing  $\text{Ca}^{2+}$  ions from the polysaccharide biofilm matrix, leading to a faster dismantlement thereof.

SDS was one of the preferred detergents and it is further worthwhile noting that this detergent is also a chaotropic agent. It is therefore contemplated that a chaotropic agent may be optionally added to increase the biofilm dislodging strength of the solution. Such chaotropic agents include 5 but are not limited to SDS, urea and guanidine. The chaotropic agent is also considered as an optional activity enhancer.

**Additives:**

Should the detergent used in the composition produce foam, it might be desirable to add an anti-foamer. Also, a dye might be added to the 10 compositions of this invention for easy monitoring of the extent of rinsing. Further, flavors or scents may be added to provide a pleasant taste or smell to surfaces to be cleaned.

Fresh water lines supplying dental instruments are of a very small diameter, which excludes the possibility of scrubbing. This would not be the 15 case for dentures, surfaces or tubings of larger diameter. The compositions of the present invention have the advantage of showing efficient decontamination in the complete absence of scrubbing in a convenient time of decontamination. The present invention is not only useful for dental instruments or prostheses. It will become obvious that it is intended for other 20 applications, e.g. cleaning or decontaminating any type of tubing or container on the surface of which microorganisms are adsorbed and form a biofilm. In such other applications, scrubbing or any other mechanical aid is not at all excluded. Should these compositions be used in pipes of a larger diameter and length, for example, wherein a non-cost effective large volume of 25 cleaning solution would be needed to fill the pipes, it is possible that a mechanical action would help in the action of the solution. A mechanical aid, when envisaged, would help in reducing the duration of cleaning and/or in spreading the cleaning solution on a surface. It is further not excluded to add a vehicle allowing the cleaning solution to stay in contact with the surface to 30 be decontaminated as long as possible. This is referred to as an adhesion enhancer. Some or all of cleaning solution components might be delivered in separate vials, in concentrated or in solid forms, to be admixed in the final reconstitution water volume. This could reduce the handling and storage of large volumes of decontaminating solutions.

35 **Minimal and Optimal Effective concentrations of components**

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Detergents:

SDS has been tried at a final concentration of 0.15% (w/v) and did work well within one hour. So, SDS certainly can be as low as about 0.1% when the duration of treatment may last about one hour or more. The most 5 preferred SDS concentration was 1% which achieved a perfect cleaning efficacy within one hour. Any detergent at a concentration as potent as at least about 0.1% SDS is within the scope of this invention. For example, the following detergents and concentrations have been tried with success:

10        -        SDDD 0.015% - 1%,  
              -        SCS 0.3% - 1%,  
              -        Tween 20™ 4%, and  
              -        CPC 0.5%-1%, and  
              -        CPB 0.5%-1%.

15 So, detergents of all classes: non-ionic, anionic and cationic, have been all successful in removing biofilms, and this invention should not be limited to the tested five detergents.

Salt-forming acids:

20 Mandelic acid has been tried with success in a concentration extending from 1% to 10%. Besides that, acetic acid has been tried in a range of concentrations 0.1% to 1% and was very efficient. Further, a plurality of acids (1%) may substitute for mandelic acid 1% (see Table 1). It is therefore contemplated that acids can be used in a minimal concentration of about 0.1% at a salt-forming pH. Preferred acid concentration is 1% for rapidly acting solutions, with reference to mandelic acid. Any acid capable of 25 forming salts at the working pH, in concentrations equipotent to at least about 0.1% mandelic acid, depending on the desired contacting time, is within the scope of this invention. Although a pH of about 5 has been tested, it is readily apparent to a skilled reader that the pH of the solutions is not restricted to that value. Indeed, when CPC was the used detergent, a solution having a 30 basic pH (namely pH 7.5) was preferred.

Cleaning Enhancers:Chelators:

Tetrasodium EDTA (0.25% - 1%) has been tried with a certain degree of success against biofilms. Any chelator in a concentration equipotent to the 5 above concentrations of EDTA is within the scope of this invention. It is worthwhile noting that HEEDTA has been used in the acid form (0.3%) and was good when another salt forming acid: acetic acid, was at a concentration of 0.1% to 1% and when the pH was brought from 2.42 to 5.0. So, chelator salts can be used or chelator acid precursors can be used in salt forming 10 conditions. It is recalled that the chelator is an optional component; it is used to increase the cleaning strength of the solution. Its function is mainly to capture divalent ions such as  $\text{Ca}^{2+}$  which are involved in EPS integrity.

Chaotropic agents:

SDS has a dual action as a detergent and a chaotropic agent. Since 15 a plurality of non-chaotropic detergents may substitute for SDS, the chaotropic activity is not considered essential to the claimed compositions. However, since SDS was one of the preferred detergents, it is contemplated that a chaotropic agent may be useful, as an optional component, in increasing the cleaning strength of the solution. Any chaotropic agent having 20 the potency of in a concentration of at least about 0.1% SDS is within the scope of this invention.

Bactericides:

When it is desirable to complete the cleaning solution with a bactericidal activity, especially in the medical field, a bactericide can be 25 added in an effective concentration. It is recalled that bactericides alone are less effective against biofilms than against planktonic microorganisms. However, when bactericides are combined to a detergent/salt solution, or contacted with surfaces thereafter, they are capable of killing microorganisms 30 which are retrieved as planktonic organisms and no longer organized as a biofilm, due to the detergent/acid/salt effect. Povidone-iodine 10%, mandelic acid 1%, sodium benzoate/salicylate 2%/2%, hydrogen peroxide 5%, sodium hypochlorite 0.5%, phenol 0.1% and CPC 0.1%-1% with or without ethanol have all been tried with success; which indicates that any bactericide may be 35 added in the cleaning solution in so far as the selected bactericide has a killing activity against the populations of microorganisms to eliminate.

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Amongst the above-listed bactericides, we have preferred mandelic acid, hydrogen peroxide, sodium hypochlorite, phenol, CPC, and CPB because these bactericides qualify as tuberculocides; they are efficient against highly resistant *Mycobacterium* species and they have a large 5 spectrum of efficiency against microorganisms. Table 1 shows that hydrogen peroxide really killed the bacteria, which translated into a quasi-total absence of growth after treatment.

Dismantling the exopolysaccharides (EPS) present in the biofilms:

EPS exist in more or less ordered forms in natural environment. Many 10 bacterial EPS appear to adopt a double helicoidal configuration and the association of the double helices is facilitated by ions (such as  $\text{Ca}^{2+}$ ) and by water molecules. The physical properties of EPS and hence of biofilms may be influenced by the presence of free anionic groups (uronic acids, phosphate groups, pyruvate ketals or succinyl half esters). Hydrogen bonding 15 involving exposed hydroxyl groups can also be significant. Localized hydrophobic regions may also exert influence. Therefore interacting with the ions involved in the maintenance of cohesive biofilms is a target for the dismantlement thereof. It has been suggested that excess  $\text{Na}^+$  may exchange with  $\text{Ca}^{2+}$  and that local proton gradients may convert the salt form 20 of the EPS to the proton form, again altering its properties. (I.W. Sutherland in "Biofilms, Community Interactions and Control" ed. J. Wim-Penny, P. Handley, P. Gilbert, H. Lappin-Scott and M. Jones. Third Meeting of the British Biofilm Club, Powys, Sept. 1997).

Even if it may have been envisaged that changes in the ionic 25 environment of a biofilm may alter its integrity, no one has ever come up with satisfactorily performing solutions, or when such solutions exist, there has been no teaching of using them for removing biofilms and this, without any mechanical aid. The present invention relates to compositions and methods 30 minimally comprising or making use of salts or salt-forming acids and a detergent. Preferably, a salt-forming acid is used to create an equilibrium between acids and salts, both of which being involved in attacking the biofilm components. The detergent appears to have a synergistic effect in 35 solubilizing the components and in exposing EPS sub-layers which, in turn, become attackable by acids and salts. The present ingredients when combined are very efficient and achieve complete removal of biofilms.

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Bactericidal agents, when added, become efficient against biofilms, because the biofilm becomes disorganized and is no longer impermeable to the anti-microbials.

**Towards making a cost-effective solution:**

5        Using H<sub>2</sub>O<sub>2</sub> in water lines is not ideal because of a consequent oxygen pressure building therein. As a promising alternative to hydrogen peroxide, we tried CPC and CPB, which are good and equivalent detergents as well as good bactericides. CPC has been preferred over CPB, because of its lower cost. CPC is a bactericidal detergent normally used at concentrations ranging  
10      from 0.01% to 2% (w/w). The best biofilm dislodging activity was found at concentrations higher than about 0.5%. Amongst the acids listed above, four of them did not quantitatively decrease the biofilm dislodging capacity of a solution also comprising CPC 0.5%, EDTA 1% at pH 7.5; these acids were glycolic, fumaric, citric and phosphoric acids at 1% concentration. The biofilm dislodging efficiency of these solutions was very good. It is believed that other components could be admixed thereto to bring this efficiency to perfect.  
15      The bactericidal activity against *Mycobacterium gordoneae* of a solution comprising CPC 0.5%, EDTA 1%, mandelic acid 1% (all w/v), at pH 7.5, was compared to CEPACOL™, a positive bactericidal control. A growth diminution of 85% has been observed with our solution, while CEPACOL™ inhibited the same by almost 100%. CEPACOL™ comprises CPC and ethanol. CEPACOL™ is a good bactericide but not a good biofilm dislodger.  
20      We believe that anyone of our solutions comprising CPC 0.5%, EDTA 1% and any of glycolic, fumaric, citric, phosphoric acids 1%, at a salt-forming pH, could be complemented with ethanol as well as with anyone of the above-listed enhancers. For example, the biofilm dislodging capacity is very good with SDS which is a detergent and a chaotropic agent. It is not excluded that SDS could be added to CPC, both of them being combined for their detergent action and for their chaotropic and bactericidal actions, respectively. The  
25      concentrations of SDS and CPC could even be reduced if such a reduction is not detrimental to the biofilm dislodging efficacy. Ethanol may be added for its enhancing efficiency on the tuberculocide activity of our solutions. Any buffering agent necessary to bring the pH at a proper value can be added.  
30      One of our goals is further to provide the most cost effective solution for biofilm removal (and destruction). It would be advantageous for all or the  
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majority of the components of our solutions to be in a solid form, with or without buffering agents, with or without additional bactericidal components. Using salts of organic acids and solid detergents can be advantageous because they may have a longer shelf life, they require less storage space,  
5 and they all be extemporaneously admixed to a determined volume of tap water. Ethanol may be optionally provided in a separate container if desirable. Alternatively, pellets can be provided in lieu of a powder. For the purpose of providing a denture cleaner, for example, a rapidly disintegrating pellet comprising all the solid ingredients and proper excipient can be  
10 produced.

It is further not excluded to add adhesion enhancers at more or less elevated concentrations. When the compositions are to be used in water lines of low cross-section the adhesion enhancers which frequently increase the solution viscosity should be in a rather low concentration. Adhesion  
15 enhancers such as glycerin or Pluronics would be added to increase the propensity of the components to come in close contact with the biofilm, when desirable.

Although the present invention has been described herein above by way of preferred embodiment thereof, these embodiments can be modified  
20 at will without departing from the spirit and the nature of the subject invention. These modifications are within the scope of this invention as defined in the appended claims.